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ORIGINAL ARTICLE

A new species of the genus *Girardia* (Tricladida: Dugesiidae) from China

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Abstract The genus *Girardia* (Tricladida: Dugesiidae) was recorded in China for the first time. A new freshwater turbellarian species, *G. sinensis* Chen & Wang, **sp. nov.**, is described, which was collected from Xinghu Lake, Zhaoqing, Guangdong Province, China. Observation and analysis of the new species was conducted on morphology, habits, reproduction, histochemistry and molecular phylogenetics. In addition, the results indicated that morphological differences of $AChE^+$ nerve structure can be used as a distinguishable character for species identification of Dugesiidae.

Key words Planarian, new species, China, biological characteristics, phylogenetics.

1 Introduction

According to the animal geographical fauna division, the Chinese mainland stretches over Palearctic Realm and Oriental Realm, and it is rich in species diversity. However, studies on taxonomy of turbellarian in China are rare. At the end of the 1900s, only 16 species of turbellarians from order Tricladida were recorded as following: *Dendrocoelopsis lactea* Ichikawa & Okugawa, 1958; *D. sinensis* Liu, 1997; *D. Suifenhensis* Liu, 1997; *Bdellocephala* sp. Liu, 1990; *Phagocata vivida* Ijima & Kaburaki, 1916; *Polycelis nyingchica* Liu, 1994; *P. xigazensis* Liu, 1994; *P. sinensis* Liu, 1995; *P. lhunzhubica* Liu, 1995; *P. wutaishanica* Liu, 1996; *P. jinglensis* Liu, 1996; *P. jingyuanica* Liu, 1996; *P. koslowi* Kenk, 1974; *Seidlia hamica* Liu, 1996; *Dugesia japonica* Ichikawa & Kawakatsu, 1964; *D. ryukyuensis* Kawakatsu, 1976. Most of these species were found in Palaearctic Realm. Research on planaria diversity in South China, belonging to Oriental Realm, is relatively few.

So far, there are only 2 species of Dugesiidae recorded in China, i.e. *Dugesia japonica* Ichikawa & Kawakatsu, 1964 and *Dugesia ryukyuensis* Kawakatsu, 1976, which were found in Taiwan, Hongkong and Zhejiang Province in China.

South China is an evergreen tropical-subtropical region, with high temperature and rainfall, which is densely covered with rivers and streams. It is also rich in plants and species diversity of freshwater planaria. There were 29 species has been recorded in recent 10 years in this area, including 1 species of Tricladida (Yu *et al.*, 2013), 18 species of Rhabdocoela (Lai *et al.*, 2013; Lu *et al.*, 2013; Wang & Deng, 2006; Wang & Li, 2005; Wang & Sun, 2011; Wang & Wu, 2005a, 2005b, 2008; Wang, 2004; Xia *et al.*, 2014; Zhang *et al.*, 2010; Zhang *et al.*, 2014), 6 species of Macrostomida (Wang & Luo, 2004; Wang *et al.*, 2004; Wang, 2005; Zhao *et al.*, 2011), 2 species of Prolecithophora (Gao *et al.*, 2011; Ma *et al.*, 2014), 1 species of Lecithoepitheliata (Peng *et al.*, 2007), 1 species of Acoela (Sun & Wang, 2014). But there is no record of Dugessiidae in Guangdong Province.

In the paper, a planarian belongs to the genus *Girardia* was reported and collected from Xinghu Lake, Zhaoqing,

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Guangdong Province, China. The reproductive behavior, individual development, histology and acetylcholinesterase (AChE) histochemical localization of the species was observed and analysed. It was identified as a new freshwater turbellarian species, *G. sinensis* Chen & Wang, **sp. nov.** The genus *Girardia* (Tricladida: Dugesiidae) was recorded in China for the first time.

2 Materials and methods

2.1 Material collecting

Take stones from the bottom of lake, wash its underside, collect the water, sedimentate about 5 min，then keep the bottom residues for separation. The collected materials were transferred to a 5 L glass bowls, and fed with pork liver slice every day. After an hour, take out the rest of the liver. Change the water every 5 days. Then selected 3 mature individuals in a culture dish, and observed them once a day. Record the species' spawning time, egg numbers, incubation period, spawning cycle and larvae numbers, and measure the size of egg, larva and mature individual. Specimens were killed by 1% hydrochloric acid, then fixed for 20 h in Bouin's fluid, embedded in synthetic paraffin, cut into slices with the thickness of 5 μm vertically and horizontally, stained by H.E method. The AChE histochemical localization method was the same approaches as Zheng *et al.* (2011). All these specimens were observed under a microscope Olympus BX51, photographed by a DP 72 digital dedicated camera, and measured by DP 2-BSW software dedicated for measurement. Figures and photographs were scanned and edited by Adobe Photoshop® version 7.0. The biological patterns were drawn according to the digital photographs. Type specimens are deposited in IZCAS, Beijing, China.

2.2 DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA was extracted from 5 randomly picked and starved individuals (marked as *Girardia sinensis* sp.1–5) using E.Z.N.A.™ Mollusc DNA KIT (Omega, Norcross, GA, USA). The DNA was stored at -20℃ for later use. A pair of specific primers (COI480F: GCTCATGGTTTARTWATGATTTTYTT; COI385R: GWGCAACAACATARTAAGTATCAT) were designed based on the sequence of the COI gene encoding cytochrome c oxidase subunit I (Lázaro *et al.*, 2011; Pongratz *et al.*, 2003). The polymerase chain reaction (PCR) was carried out using a Premix Ex Taq™ Hot Start Version (TaKaRa, Otsu Japan). The PCR amplicants were recovered and purified using a Gel DNA Extraction Kit (Newtopbio, Shenzhen, China), ligated into pUCm-T vector (BBI, Toronto, Canada), and then transformed into the competent cells of *Escherichia coli* Top 10. Target genes of positive clones were sequenced by Beijing Genomics Institute (BGI, Shenzhen, China). To obtain homologous sequences, a similarity search of the target nucleotide sequence was performed using the NCBI blastn option. The multiple sequences alignment was conducted among these COI sequences from the families Geoplanoidea and Planarioidea (Table 1) using DNAssist (version 2.0, Los Alamos, NM, USA). *Dendrocoelum lacteum* of Planarioidea was chosen as the outgroup. The phylogenetic tree was plotted using MEGA software (version 6.0) (download from: www.mega.software.net). Maximum-parsimony (MP) and maximum-likelihood (ML) methods with Tamura-Nei model were used to show the inferred evolutionary relationships among these species. Due to the low homology and recovery rate of COI nucleotide sequences among the species within the order Tricladida, this study selected 420 bp sequences to root the tree.

The following abbreviations used in the text and figures.

PLA-G. Platyhelminthes-*Girardia*;

IZCAS. Institute of Zoology, Chinese Academy of Sciences;

$AChE^{+}$. Acetylcholinesterase positive reaction;

bs. bursa stalk;

ca. common atrium;

cb. copulatory bursa;

cg. cerebral ganglion;

e. eye;

ed. ejaculatory duct;

go. gonopore;

lnc. lateral nerve cord;
ma. male atrium;
o. ovary;
od. oviduct;
p. penis;
pd. posterior diverticulum;
ph. pharynx;
pmc. penis muscle cord;
pnc. pharyngal nerve cord;
pp. penis papilla;
ps. pseudopodium;
sg. shell glands;
sv. seminal vesicle;
t. testis;
tn. transverse nerves;
vd. vas deferens;
vm. ventral muscle;
vnc. ventral nerve cord.

Table 1. Nucleotide sequences selected for phylogenetic analysis.

Family	Species	GeneBank acc. no.	References
Planarioidea	*Dendrocoelum lacteum*	AF178312.1	Riutort *et al.*, 2001
Geoplanoidea	*Bipalium adventitium*	AF178306	Riutort *et al.*, 2011
	Bipalium multilineatum	HM346600	Huh & Jo, 2010
	Dugesia japonica	AB618487	Sakai & Sakaizumi, 2012
	Dugesia ryukyuensis	AB618488	Sakai & Sakaizumi, 2012
	Girardia tigrina	AF178316	Riutort *et al.*, 2001
	Novibipalium venosum	HM346599	Huh & Jo, 2010
	Girardia sinensis sp.1	KP091895	Present work
	Girardia sinensis sp.2	KP091891	Present work
	Girardia sinensis sp.3	KP091892	Present work
	Girardia sinensis sp.4	KP091893	Present work
	Girardia sinensis sp.5	KP091894	Present work

3 Systematics

Family Dugesiidae Ball, 1974

Genus *Girardia* Ball, 1974

***Girardia sinensis* Chen & Wang, sp. nov.** (Figs 1–21)

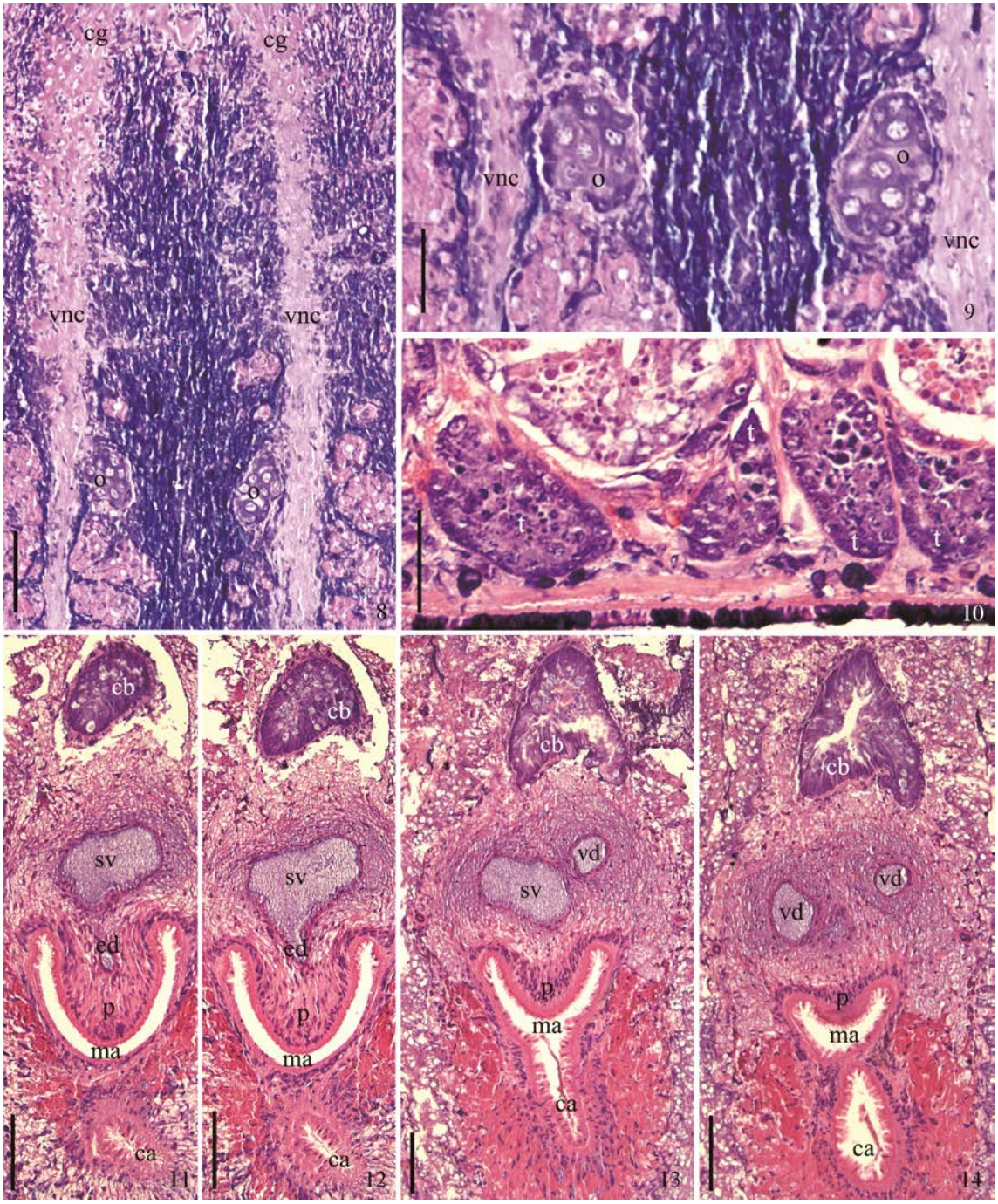
Locality. The animals were collected from the ventral part of stones underwater (about 1.2–1.5 m in depth) in Xinghu Lake, Zhaoqing, Guangdong Province, China (23°04′33″N, 112°28′43″E). Xinghu Lake appears karst topography, and the lake area is 8.23 km^2. There are many granite hills and karst caves in the lake, which forms lake wetland naturally. No aquatic plants were found nearby, and the lake belongs to the freshwater habitats. The shoreside is the urban residential area, where domestic sewage discharging into the lake.

Materials. PLA-0080, serial section vertically; PLA-0081, serial section horizontally; PLA–0082 –89, the whole body is fixed in 10% formaldehyde.

Figs 1–7. *Girardia sinensis* Chen & Wang, **sp. nov.** 1–4. Mature. 1–2. Mature individuals, dorsal view. 3. Mature individuals, ventral view. 4. Pseudopodium. 5. New egg. 6. Egg clusters and the incubative individual. 7. Immature individual, dorsal view. Scale bars: 1–3 = 1 mm; 4–7 = 500 μm.

Etymology. The specific name is referring to its type locality, China.

Description. External morphology. Body size of living individuals ranges from 14.2 to 15.2 mm in length and 3.4 to 4.2 mm in width. The animal appears flat, and is covered by massive black and white spots dorsally, similar to granite pattern. Both sides of triangle-shaped head have triangle-shaped auricular flanges, which can stand up ranging from 15 to 100 degrees, which are unobvious in fixed specimens (Fig. 3). Posterior back of auricular flange have obvious oval unpigmented area (Figs 1–2). A pair of kidney-shaped eyes lies between the auricular flanges. Fan-shaped unpigmented area placed at the lateral part of eyes (Figs 1–2). Mouth lies near the middle of ventral side of the body. Gonopore locates behind mouth at 1/3 rear part of the body (Fig. 3). Both sides of the body have obvious cement glands thar ventrally

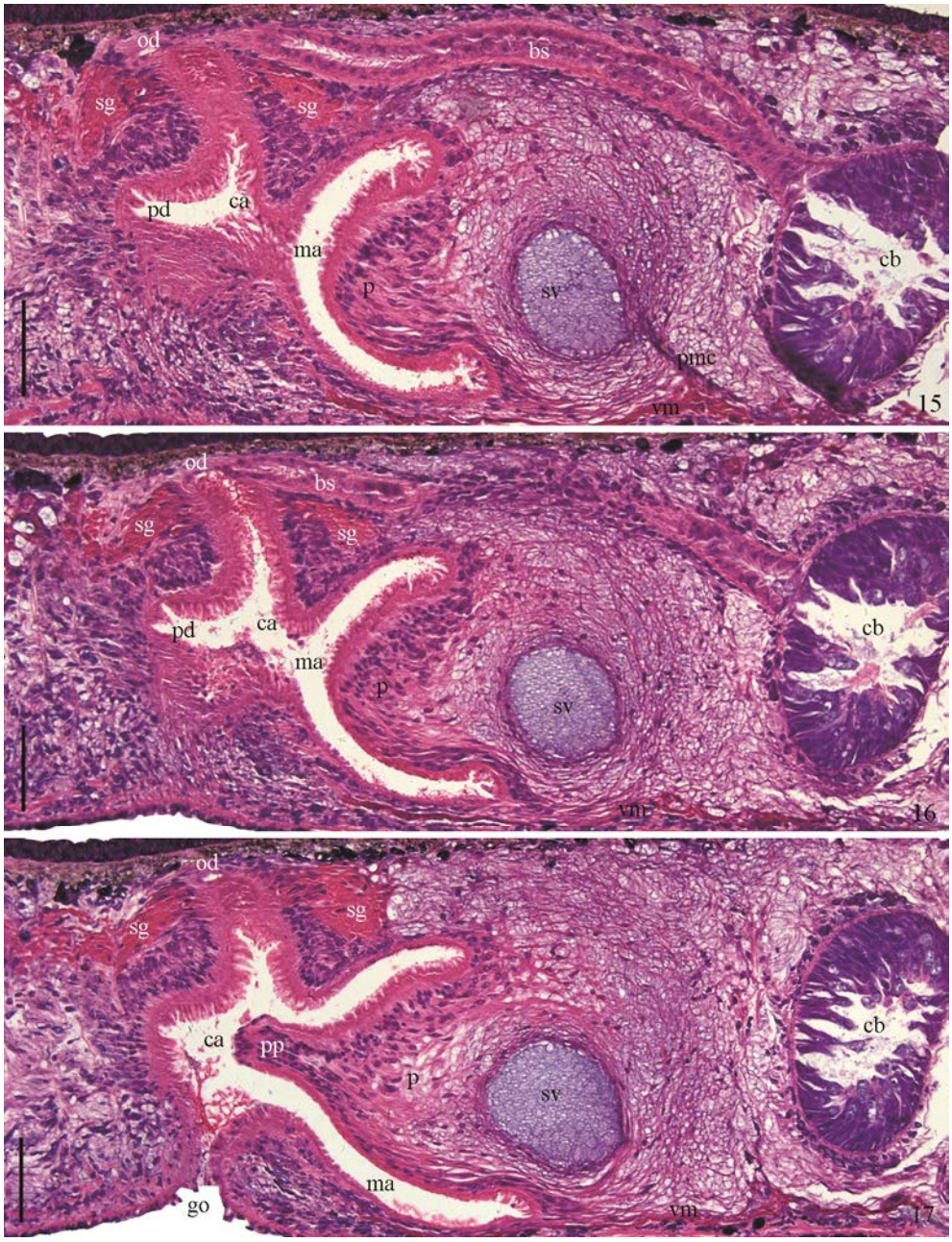


Figs 8–14. *Girardia sinensis* Chen & Wang, **sp. nov.** 8–10. Body anterior part close to ventral side. 9. Ovaries. 10. Testes. 11–14. Sagittal section copulatory apparatus. Scale bars: 8, 11–14 = 100 μm; 9–10 = 50 μm.

distributed, when in stationary state, pseudopodium appear at both sides, and vary in shape (Fig. 4).

Habits. Animals moves slowly, wriggles on the wall or close to the bottom of culture dishes when foraging. They prefer to cluster in the underside of stones. Also, the species have strong pollution-resistance ability, which can adapt to nitrogen and phosphorus eutrophic waters, and were fed with pork liver under artificial cultivation. When prey, they swim to the upward side of foods, darted out its pharynx from ventral side of body to encase food and ingest, then drift away or adsorbed on the surface food.

Reproduction. Collected individuals were cultivated indoor under room temperature (25±1°C). They showed sexual reproduction after a week, then copulatory apparatus disappeared 2 months later. After 9 months, they appeared sexual reproduction again, and conducted transverse fracture asexual reproduction during other periods. Mature individuals clustered in the ventral side of underwater stones to lay eggs. When laying eggs, gonopore excretes mucus to the surface of stones, and raised up gradually, then the mucus converted into egg stalk (0.43–1.00 mm in length). Finally, eggs are attached



Figs 15–17. *Girardia sinensis* Chen & Wang, **sp. nov.**, longitudinal section of copulatory apparatus. Scale bars = 100 μm.

to the egg stalk, which looks similar to cherry shape as a whole. The size of spherical-shaped eggs differs from each other produced by different individuals (0.77–1.36 mm in diameter). The just-laid eggs appear light red, then bright red few days later, and dark red at last (Fig. 5). The specimens stop eating after laying eggs, and incubate around egg stalk about 18 d (18±3d, *n*=36) until the larvae hatched (Fig. 6). Each egg hatches one larva, and its appearance is similar to mature individual, uniformly distributing black and white oval spots dorsally (Fig. 7).

Digestive system. Digestive system consists of mouth, pharynx and intestines. Mouth opens at the middle part ventrally. Cylinder-shape pharynx (18 mm×0.33 mm) with thick pharyngeal musculature lies in the central part ventrally, elongated longitudinally to the rear. The surface of pharynx is covered by brown pigmentation. The base of pharynx connects to 3 intestinal branches, and the middle one stretches to the anterior part of eyes, the other two extend to the posterior end of the body.

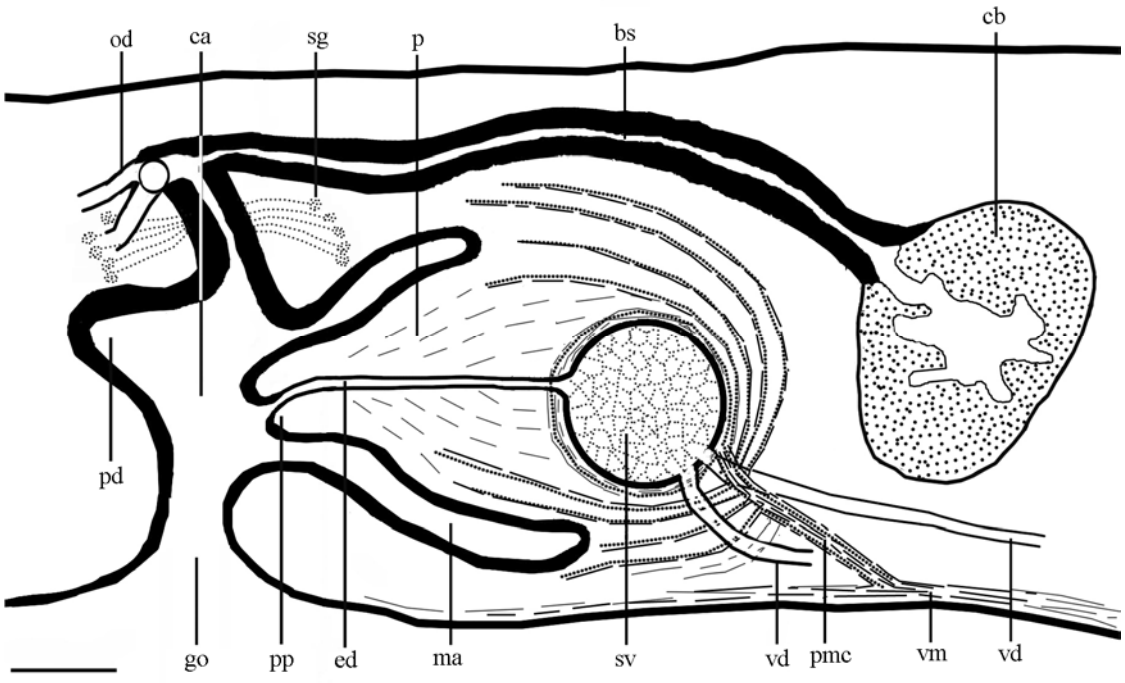


Fig. 18. *Girardia sinensis* Chen & Wang, **sp. nov.**, sagittal reconstruction of copulatory apparatus. Scale bar = 100 μm.

Reproductive system. Hermaphrodite, with one gonopore. Female reproductive systems are composed of ovaries, oviducts, copulatory bursa, bursa stalk and common atrium. A pair of oval ovaries (80 μm×130 μm) lies in the anterior inner side of ventral nerve cord (Figs 8–9). Extending from ovaries, oviducts elongate backward to gonopore dorsally, then join in the junction of bursa stalk and common atrium (Figs 15–18). Sphere-shaped copulatory bursa placed right behind pharynx, and joints common atrium through bursa stalk (Figs 11–18). Pipe-shaped bursa stalk (740 μm×55 μm) lies above seminal vesicle, extending to common atrium dorsally. Common atrium connect with the outside surroundings through gonopore (Figs 15, 18). Male reproductive systems consist of testes, vas deferens, seminal vesicle, penis, male atrium. More than 20 pairs of oval testes (60 μm×90 μm) stretch longitudinally along both sides of the body (Fig. 10). Each testis extends out vas deferens, and elongate backward to seminal vesicle. Irregular net-shaped seminal vesicle locates right behind copulatory bursa and under bursa stalk, and the solid inner side is filled with spermatozoa. An obvious penis muscle cord lies below seminal vesicle, incline to body ventral part, and link to ventral muscle. Penis muscle cord give out 4–5 muscle fiber bundles around seminal vesicle. Each muscle fiber bundle gives out numerous muscle fibers, interconnecting into a mesh structure (Figs 11–18). Penis consists of ejaculatory duct and penis papilla. Extending from seminal vesicle, ejaculatory duct opens to male atrium through penis papilla, and joins with common atrium. Nipple-shaped penis papilla locates just behind seminal vesicle, and connects with the outside surroundings through gonopore. The periphery of penis is male atrium. The rear muscle behind common atrium sink backward to form an obvious posterior diverticulum. The terminal joints of common

atrium and bursa stalk are distributed with shell glands densely (Figs 15–16, 18).

AChE$^+$ nerve structure. AChE+ nerve structure appeared copper red after histochemical localization, and it consists of cerebral ganglion, ventral nerve cord, lateral nerve cord and transverse nerves (Figs 19–21). The control specimens showed a negative reaction. Cerebral ganglion are formed by two-sided arc-rod-shaped nerves, they joint in the anterior end and

Figs 19–21. *Girardia sinensis* Chen & Wang, **sp. nov.**, AChE$^+$ nerve structure. 19. Mature individuals, ventral view. 20. Head. 21. Body, middle part. Scale bars: 19 = 200 μm; 20–21 = 100 μm.

appear reverse V shape. Both sides of cerebral ganglion extend backward to form ventral nerve cord ventrally. Cerebral ganglion sent out about 11 radial branch nerves to connect with lateral nerve cord, then elongate to the body side. Two-sided cerebral ganglions are connected by densely no branching transverse nerves (Fig. 20). It is difficult to define the boundary between cerebral ganglion and ventral nerve cord. Ventral nerve cord lies in both sides of the body with a diameter of about 20–50 μm ventrally. In the anterior end, they join with two-sided cerebral ganglion respectively and extend along the body to the posterior end connecting by arc-shaped nerves. The point where ventral nerve cord sent out branch nerves shows obvious bulge, which contains nerve cells inside. The branch nerves emitted by the right and left ventral nerves connect with lateral nerve cord transversally (Fig. 19). Lateral nerve cord locates in both sides of the body and parallel ventral nerve cord. They come from the first pair of branch nerves and extend backward along the body side to the terminal end of ventral nerve cord (Figs 19–20). In the central part of the body, there are 1–2 incomplete longitudinal nerves between lateral nerve cord and ventral nerve cord (Fig. 21).

Phylogenetic analysis. The phylogenetic trees generated using maximum-parsimony (MP) and maximum-likelihood (ML) analyses were shown in Fig. 23 and Fig. 24, respectively. Both results suggested that the COI sequences from *Girardia sinensis* sp. 1–5 formed a clade with 100% bootstrap probabilities. In addition, these sequences also formed a clade with 100% bootstrap probabilities with the sequence of *Girardia tigrina*. It should be noted that *Dugesia japonica* and *Dugesia ryukyuensis* formed a 100% probabilities clade, suggesting that the genus *Dugesia* is likely to be a sister group of the genus *Bipalium*, *Dugesia*, *Girardia* and *Novibipalium* from the family Geoplanoidea.

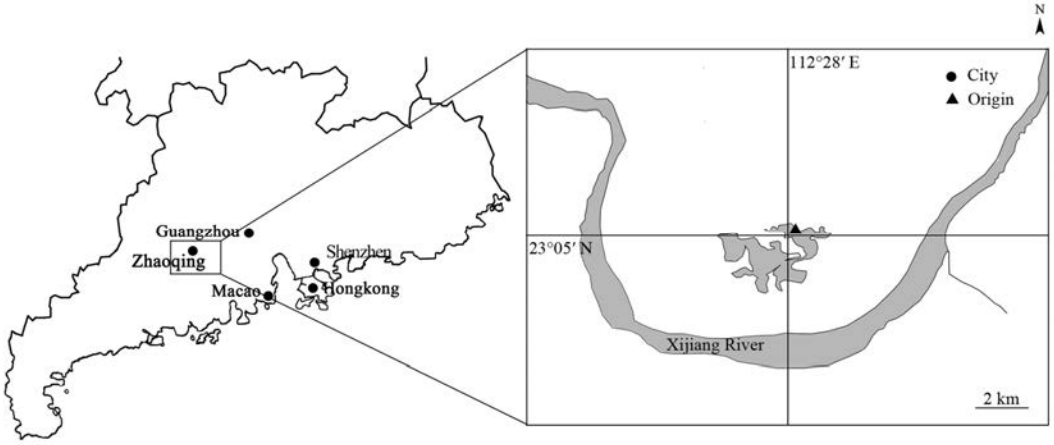


Fig. 22. *Girardia sinensis* Chen & Wang, **sp. nov.**, distribution.

4 Discussion

4.1 Classification

At the end of 2013, 46 species of the genus of *Girardia* have been reported worldwide (Tyler *et al.*, 2006–2012). The type specimen of *Girardia sinensis* Chen & Wang, **sp. nov.** inhabits in the lake bottom (1.2 m in depth), and the lake is lightly eutrophicated. For the first time, the genus *Girardia* (Tricladida, Dugesiidae) was recorded in China.

The genus *Girardia* is normally characterized by following features: black and white spots scattered throughout the back; a triangle-shaped head with two sharp auricular flanges; barrel-shaped pigmented pharynx; highly-developed bursa stalk; some own an obvious posterior diverticulum and pseudopodiums with cement glands ventrally distributed.

Besides the typical features of genus *Girardia*, the type specimen of *G. sinensis* Chen & Wang, **sp. nov.** is similar to *G. tigrina* (Girard, 1850). *G. tigrina* are widely distributed in freshwater. It has a pair of ovaries and many testes distributed longitudinally at both sides. Copulatory bursa extends backward dorsally through bursa stalk to link common atrium, and the right and left oviduct join together in the end of bursa stalk. A pair of gourd-shaped seminal vesicle placed in front of penis. After terminal expanding of vas deferens, seminal vesicle links to ejaculatory duct, which is wrapped by penis. The

periphery of penis is male atrium, which is connected with common atrium. Cyclic and radial muscle fibers cross distributed under bursa stalk (Kawakatsu *et al.*, 1992).

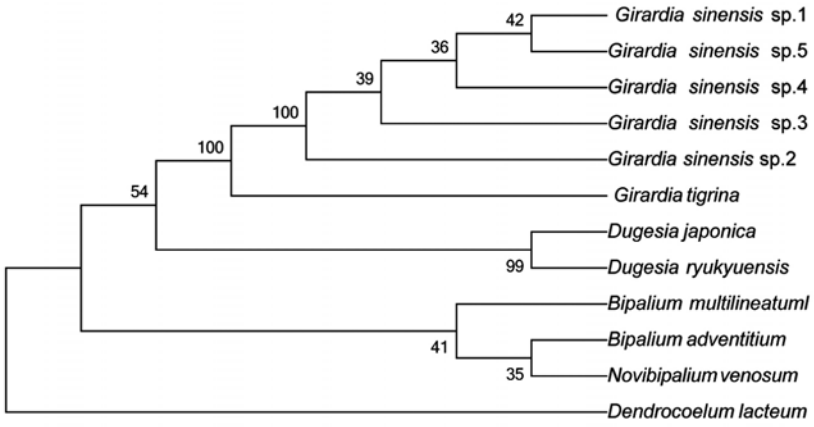


Fig. 23. Phylogenetic tree generated based on Maximum-parsimony (MP) analysis of partial sequence of COI gene. *Dendrocoelum lacteum* (Planarioidea) was chosen as outgroup.

As for *G. sinensis* Chen & Wang, **sp. nov.**, the dorsal part is distributed with irregular black spots, and both sides have obvious cement glands. The two sides also appear pseudopodium in different shapes under stationary state. Copulatory bursa extends backward to link common atrium through bursa stalk. Oviducts join in the junction of bursa stalk and common atrium. There is a sphere-shaped seminal vesicle lies at the base of penis. The short ejaculatory duct connects to seminal vesicle directly, and the front end of ejaculatory duct is not expanded. The rear muscle fibers behind common atrium sink backward to form an obvious posterior diverticulum, which is a specific characteristic of genus *Girardia*. Seminal vesicle is surrounded by muscle fibers at the base of penis, and there is no radial-shaped muscle fiber. An obvious penis muscle cord lies between the base of penis ventrally and ventral muscle. From these above, there exist significant differences between type specimens of *G. sinensis* Chen & Wang, **sp. nov.** and *G. tigrina*. Therefore, we concluded that *G. sinensis* Chen & Wang, **sp. nov.** is a new species of the genus *Girardia*.

4.2 AChE$^+$ nerve structure characteristics

The study shows that the nerve structure of *Girardia sinensis* Chen & Wang, **sp. nov.** consists of cerebral ganglion, ventral nerve cord, lateral nerve cord and transverse nerves. Two-sided cerebral ganglion connect each ventral nerve cord respectively, and lateral nerve cord sent by the first pair of cerebral ganglion joint ventral nerve cord at the posterior end of the body.

According to previous studies, *Dugesia japonica* displayed 9 sent from cerebral ganglion using immunohistochemical method (Umesono & Agata, 2009), *Girardia tigrina* showed 10 nerves using neuropeptide F (NPF) and serotonin (5-HT) method (Reuter *et al.*, 1995), and the type specimens of this study revealed 11 nerves.

The planaria specimens for Zheng's study were collected in Wenshan Lake, Shenzhen of Guangdong Province (Zheng *et al.*, 2011). The specimens were significantly different from *Dugesia japonic*a on morphology, but they appear quite similar on habitat, appearance and behavior, therefore, its classification status remains to be further identified. After careful comparison, the major differences between *Girardia sinensis* Chen & Wang, **sp. nov.** and Zheng's specimens were as follows: 1) in Zheng's specimens, two-sided cerebral ganglion were tightly ctionnected, but in *G. sinensis* Chen & Wang, **sp. nov.**, the cerebral ganglions were not closed completely; 2) In Zheng's specimens, the nerve system showed a three-dimensional network structure. Dendritic transverse nerves locate among ventral nerve cord and connect to lateral nerve cord, i.e. numerous transverse nerve branches. But in *G. sinensis* Chen & Wang, **sp. nov.**, there are obvious transverse nerves emitted by ventral nerve cord, but the branch nerves are rare. Transverse nerves connect to lateral nerve cord, which resemble a trapezoid structure as a whole. Based on the above comparison, for the first time we conclude that morphological differences of AChE$^+$ nerve structure can be used as a distinguishable character for species identification of Dugesiidae.

The species of the genus of *Girardia* is widely spread in North America and South America, which belong to

Neotropical Realm and Nearctic Realm repectively, considering the animal geographical fauna division. Besides, the species were also found in Ireland, Italy, Germany and France (belonging to Palaearctic Realm), Australia and New Zealand (belonging to Australian Realm) and Japan (belonging to Oriental Realm) (Ribas *et al.*, 1989). This species inhabited in ponds, lakes, rivers and the freshwater area of mediterranean coast. *G. sinensis* Chen & Wang, **sp. nov.** was found in the underside of stones from Xinghu Lake, Zhaoqing, Guangdong province, China, which belongs to Oriental Realm (Fig. 22).

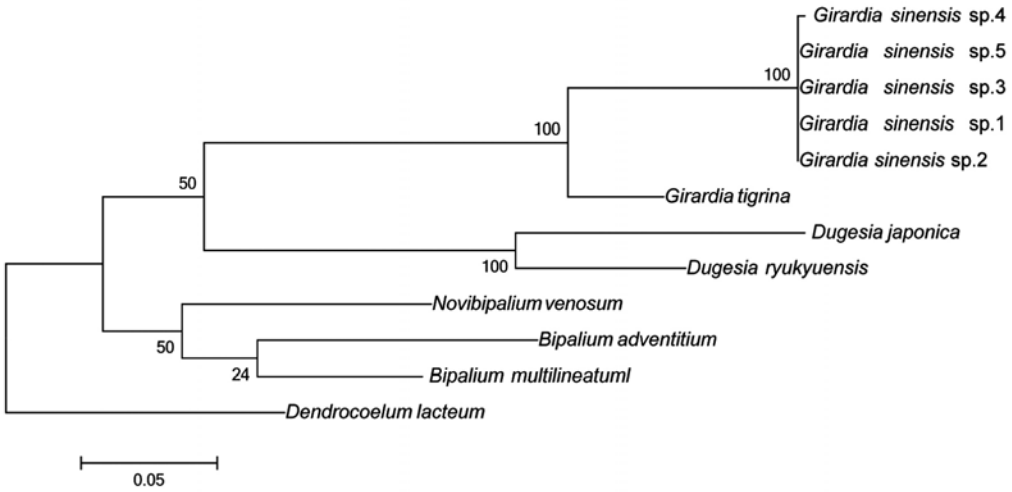


Fig. 24. Phylogenetic tree generated based on maximum-likelihood (ML) methods with Tamura-Nei model analysis of partial sequence of COI gene. *Dendrocoelum lacteum* (Planarioidea) was chosen as outgroup.

4.3 Phylogenetic analysis

The phylogenetic analyses results showed that, in both maximum-parsimony (MP) and maximum-likelihood (ML) based methods, COI sequences from *Girardia sinensis* Chen & Wang, **sp. nov.** 1–5 formed a clade with 100% bootstrap probabilities. What's more COI sequences of these five individuals and *Girardia tigrina* formed a clade with 100% bootstrap probabilities as well. Overall, these data have provided firmed molecular phylogenetics evidence that *G. sinensis* Chen & Wang, **sp. nov.** and *G. tigrina* are distinct species belong to the genus *Girardia.*

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